DETECTION OF HBV CO-INFECTION WITH HDV AND HCV IN NON HOSPITILIZED PATIENTS.

By

ISMAIL JALIL

Submitted in the partial fulfillment of the
requirements for the degree of
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in

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Department of Microbiology

Faculty of Biological Sciences

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CERTIFICATE

A thesis submitted in the partial fulfillment of the requirements for the degree of the Master of Philosophy. We accept this dissertation as conforming to the required standard.

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DEDICATION

I affectionately dedicate this effort to my honorable parents and all my teachers whose sincere prayers, cordial well wishes and matchless kindness have made me extremely successful in each and every sphere of this mortal life.

CONTENTS

LIST OF TABLES

LIST OF FIGURS

LIST OF ABBREVATIONS

AVH	Acute Viral Hepatitis
ALT	Alanine Aminotransferase
CLD	Chronic liver Diseases
CAH	Chronic Active Hepatitis
DOAJ	Directory of Open Access Journals
DNA	Deoxyribo Nucleic Acid
ELISA	Enzyme Linked Immunosorbant Assay
FHF	Fulminant Hepatic Failure
HCC	Hepatocellular Carcinoma
HMC	Hayatabd Medical Complex
IDPs	Internally Displaced Persons
LFTs	Liver Function Test
OBI	Occult hepatitis B virus Infection
OPD	Out Door Patient
SPSS	Statistical Package for the Social Sciences, Statistical Product and Service Solutions
RNA	Ribonucleic Acid
RT-PCR	Real Time Polymerase Chain Reaction
TH	Triple Hepatitis
TBE	Tris Borate EDTA
WHO	World Health Organization

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Ismail Jalil

ABSTRACT

Infection with B viral hepatitis is a critical public health issue in both under-developed and developed countries which affected approximately 3.5 billion people throughout the world and additionally ≥ 400 million are chronic carriers. In Pakistan, different kind of published work about HBV infection have been found which grossly discuss HBV issues of epidemiology and rate of prevalence. Although all these documented data reflect quite different picture of the disease. The current study briefly describe the incidence rate, risk factors correlated with HBV transmission among the male and female population of different age groups, chronic and acute status of HBV and comparison of different genetic and serological marker of HBV prevalent in KPK population. Patient sera were subjected to HBsAg, Anti-HBcroe total, Anti-HBcore IgM, Anti-HCV and anti-HDV screening by ELISA and finally HBsAg positive samples were further processed for polymerase chain reaction. Out of total 212 HBsAg ELISA positive samples 53.77% (114) are male and 46.22% (98) female. Out of 212 HBsAg positive patients 184 (86.79 %) are found positive with anti-HBcore total which indicate the rate of chronic infection. 25.0 % (n 53) are found positive for hepatitis B core IgM which reflects acute HBV infection. Similarly HBV DNA is detected in 24.52 % (n 52) of patients with active DNA replication. Although HBV co-infection with HDV and HCV is found in 9.90 % and 7.54 % of patients respectively, the highest incidence frequency of 70.75% is found in the age group of 5-30 years while a lower rate of incidence 0.94% and 5.66% is found in the age group of below 5 years and above 50+. The observed prevalence is age and gender-dependent which might be due to their increasing exposures to general risk factors. To prevent the transmission of B viral infection considerable awareness about the possible common risk factors and immunization extension is recommended. PCR based test can detect low level of viraemia in non-replicative HBV disease. The HBV co-infection with HCV and HDV is not uncommon in Pakistan that tends to increase over time.

Introduction

Hepatitis B virus infection is a major global health problem (Idrees et al., 2004), specifically in southern Europe Asia, Latin America and Africa (Li et al., 2010). People infected with B viral hepatitis are about two billion throughout the world.(Paraskevis et al., 2002), and about 400 million are suffering from chronic HBV infection (Alam et al., 2007). Pakistan falls in highly endemic region of HBV infection (Noorali et al., 2008) with at least 9 million individuals infected with B viral hepatitis and rate of infection is rising (Hakim et al., 2008). The reason for elevated number of cases may be the lack of awareness, improper facilities of health and economically poor background (Alam et al., 2007).

Hepatitis B virus belong to family member of Hepadnavirus that consist of lipid envelope, double stranded DNA with circular symmetry, icosahedral nucleocapsid and 42 nM in diameter normally called "Dane particles". Embedded proteins present in the envelope of this virus are responsible for susceptible host cell entry and attachment. HBV virus have three different types of antigens HBsAg, HBcore-Ag and HBe-Ag (Howard., 1986). Similar to retroviral reverse transcription this virus also replicates by RNA intermediate. After completion of their replication in the cells of liver which are known as hepatocytes it spreads via blood and causes viremia. Different serological and genetic markers of HBV are detected in the blood of infected people. According to WHO Fact sheet of 2004 B viral hepatitis is fifty to hundred times more virulent than Human Immunodeficiency Virus (Locarnini., 2004; who. Fact Sheet).

Hepatitis B is originally known as "serum hepatitis'' which causes an infectious inflammatory illness of the liver (Barker et al., 1996). Hepatitis Delta virus is basically RNA virus depends on the surface antigen of hepatitis B for its multiplification due to its defective nature. HBV and HDV mutually cause dual infection in the form of super-infection also called co-infection. In case of both Delta and B viral hepatitis co-infection, they cause usually serious acute liver illness and pose an increase risk for the progressive

fatal hepatitis. The Super infection of HBV with HDV results in escalating up to (80%) chronic liver disease, that further exaggerates hepatocellular carcinoma and cirrhosis of liver (Yamaguchi et al., 2002).Worldwide About 5% of hepatitis B chronically infected individuals are co infected with Delta hepatitis virus, leading to a figure of approximately fifteen million. Frequency and prevalence of HDV infection worldwide varies in different geographical regions. In Pakistan it has been estimated that the prevalence of chronic HBV carrier state is about 16 to 57 % in common population but most particularly in men with young aged residing in rural regions (Khan et al., 2011). Chronic hepatitis clinical course and sequels are varying among individuals. HBV infection results in a wide spectrum of clinical manifestation, like from carrier state with no symptoms of disease to acute infection and this acute infection becomes fatal hepatic failure, chronic hepatitis or self limiting disease. While chronic hepatitis progress to HCC and cirrhosis of liver (Zhu *et al.,* 2008).

For the classification and diagnosis purposes of HBV infection several serological markers are scrutinized. For the analysis of diagnosis, of hepatitis B infection and vaccination efficacy, five valuable serological markers are used including surface antigen of B virus, hepatitis e antigeng, Antibodies to B surface Ag, Antibodies to e antigen, and anti-HBc, total (IgG, IgM) (Chen et al., 2011).

Serological profile of HBV can be complex and varys in different stages of the disease diagnosis. Chronic hepatic inflammation induced by HBV, may result in an entangled serological profiles. The most common serological indicator of B viral infection is the presence of HBsAg whereas HBeAg is used as a supporting marker to specify active HBV infection and coexisting progressive liver disease. For the clinical assessment and evaluation of the individuals with B viral infection, HBeAg, antibodies to e antigen, DNA of HBV and HBsAg assays of the serum samples are useful. DNA of HBV presence in the serum of chronic hepatitis patients indicates that viral replication in the hepatocytes is still in progress (Rodrigues et al., 2001).

Hepatitis B infection (anti-HBe) seroconverion is usually encouraged by inhibition of HBV replication and liver disease remission (Chan et al., 2001). Variability of HBV genome results in the naturally occurring most common variants which include the precore (Pre-C) stop codon mutation (G 1896 A) that inhibits HBe-Ag production and the dual mutation in the basal core promoter (BCP) region (A1762 T, G 1764 A) can diminish HBe antigen production (Scaglioni et al., 1997). Majority of chronic HBV patients with HBe Ag negative are found with precore variants in which replication of HBV and inflammation of hepatic cells persist in the absence of HBeAg. High prevalence about 50- 80 % of individuals with hepatitis e antigen negative chronic hepatitis have been reported in Asia and Europe, with Precore and core promoter mutation.(Funk *et al.*, 2002).

The unusual circular DNA of HBV that is not completely double-stranded. Viral DNA polymerase attached to one end of complete strand. The full-length strand genome size is from 3020–3320 nucleotides and short length-strand size is 1700–2800 nucleotides long (Kay and Zoulim., 2007). Viral messenger RNA is complementary to the negative-sense. When the host cell becomes infected the incomplete strand of DNA has become fully double strand. HBV genome encodes four genes, called C, S, P, and X. The proteolytic processing of pre-core protein results in the formation of HBeAg. The protein core is encoded by gene C also called HBcAg. Gene P encodes DNA polymerase, and surface antigen (HBsAg) is encoded by gene S (Beck and Nassal., 2007). Protein encoded by gene X is responsible for the liver cancer development and its function is not fully understood although it is reported that it has a role in enhanced cell growth and inhibits molecules which are responsible for normal growth regulation (Li *et al.*, 2010).

Hepatitis B virus being endocytosed after binding to host cell surface via a receptor. Chaperones transfer viral genomic DNA into the host nucleus. Completion of fully double stranded DNA from partially double stranded occurs inside the nucleus and then changes into covalently closed circular DNA which is then used as templates of

mRNA for transcription. Similarly four viral transcripts are then produced those are further processed to produce progeny virions. The virion are then either released or re-enter to the nucleus and produce more copies after recycling (Bruss, 2007, Beck and Nassal., 2007).

Hepatitis B virus have 4 main serotypes (including ayr, adw, ayw, adr,) and have 8 genotypes (A to H). The nucleotide sequence variation of overall genome is the result of these genotypes. Due to the difference in their properties, most genotypes are further divided into subtypes (Schaefer., 2007). Different genotypes are distributed in distinct geographical location. Fluctuation in disease severity, vaccination, results of treatment, course of the disease and likelihood of complications is different between different genotypes (Kramvis *et al.*, 2005).

HBV viral genome persistence in the liver tissue of B surface Ag negative persons results in occult HBV infection. In case of OBI DNA of HBV may be apparently present in the liver (with presence or absence of DNA of in serum) in patient with serological markers of earlier infection (antibodies positive against B surface Ag and/ or core Ag) or in patients without serological markers (antibodies negative against B surface Ag and/ or core Ag) (Gutiérrez-García et al., 2011). Therefore the correlation of different serological markers with molecular diagnostic tests is prerequisite for presuming the course of chronic liver illness. We have correlated various serological markers of hepatitis B viral infection with DNA amplification of HBV and appraise its positivity in acute and chronic HBV infection.

Hepatitis B viral surface antigen preS domain binds to the host cell receptor, and gets internalized subsequently by endocytosis. IgA receptors and HBV-PreS are responsible for such interaction. After replication inside the cell, hepatitis B deregulation the normal functions of the hepatocytes. There is no authentic information regarding receptor of HBV, although there are some evidence that receptor found in the duck HBV

are carboxypeptidase D which is closely related to it (Tong *et al.,* 1999). The immune system are responsible for both damage to liver cells and viral clearance during HBV infection. While more specifically the adaptive immune response especially cytotoxic T cell play a significant role in the injury of liver during HBV infection. cytotoxic T lymphocytes killing those cells which are infected by B viral hepatitis, to eliminate hepatitis B virus and produce cytokines, which prevent viable hepatocytes from HBV infection (Iannacone *et al.,* 2007). This study briefly describe the infection frequency, risk factors associated with transmission of HBV among the women, men individuals of different age groups, chronic, acute status of HBV and comparison of different genetic, serological marker of HBV prevalent in KPK population.

OBJECTIVES

1. Comparison of Serological and Genetical markers of Hepatitis B virus.
2. To identify the Risk factor involved in Chronic and Accute HBV infection.
3. To measure the Ratio of HBV co-infection with HDV and HCV.
4. To see age and gender wise distribution of Hepatitis B virus in KPK.

REVIEW OF LITERATURE:

Alam *et al.* (2007) pointed out that the disease rate of HBV infection is constantly escalating in Pakistan. They stated that a widespread study of epidemiological data is the requirement for the clarification of true picture of HBV infection rate. A total of 1300 patients samples they selected for their study and were screened all of them for hepatitis B serological markers including HBeAg, anti-HBcAg, HBsAg and antibody to surface antigen. In order to analyze the association of these variable and patients disease status, they compared these serological markers with patients epidemiological attribute like residential area, age and socio-economic status. After screening they reported that 52 (4%) subjects were confirmed HBsAg positive, and their mean age was 23.5 ± 3.7 years. Similarly HBeAg, antibodies to surface antigen, and antibodies HBcore antigeng were 9.30%, 33.47% and 12% respectively. They further found that seropositivity rate of HBsAg was extremely associated (p = 0.03) with the residing territory indicating high infection in rural region. An inverse correlation was reported among antibodies titer against HBsAg which was decreased with the growing age. They also concluded that the prevalence rate of HBV infection is high so this indicates that educational program, masses awareness and vaccination strategy is urgently needed.

Alam et al. (2007) analyzed that HBV have eight known genotypes designated as A-H but in Pakistan no such work done on the prevalence of genotypes. Therefore, they conducted a study on the prevalence of genotypes of hepatitis B virus in the indigenous population of Pakistan. For the analysis of HBV genotyping they have tested a total of 690 individuals with EIA and nested PCR. All positive samples were further processed for multiplex-PCR by using especially designed primers to examined specific HBV genotypes (A-F). Out of 690 individuals they have screened, 110 (15.94%) of them were HBV positive, 64% of them were males and 36% females. They found that a total of 66 subjects (65.34%) were positive for genotype D, genotype B was found in 27 (26.73%), while genotype A was found in 5(4.95%) individuals. They reported that 3 (2.98%) individuals had multiple genotypes and were classified them genotypes B+D; 1(0.99%) and genotype A+B; 2(1.99%). Similarly they reported 9 (8.18%) cases samples which

were remained untypeable. They further concluded that genotype D is more common and major health problem in Pakistan, while B and C genotypes are the most prevalent genotype in Asia.

Alam et al. (2007) analyzed that the epidemiological significance of HBV genotype has been well practiced and becoming an important application constantly however, very minute data are available on mixed HBV genotype infection and their clinical concern. They concluded that transmission and acquisition of HBV infection is due to the intravenous drug abusers which is the main risk factor of this blood born viral infection however, there is no such data reported about genotyping and epidemiology of HBV in Injecting Drug Users. They screened a total of 250 individuals for HBsAg serological assay and then all the positive samples were assayed for genotype. They reported a total of 56 (22.4%) cases that were confirmed positive for surface antigen. They further classified genotype in positive samples as: 62.5% genotype D, genotype A was found in 8.92% while genotype A and D mixed infection was found in 28.57% individuals. Similarly they concluded that to minimize and control the transmission of HBV infection in high risk group especially in intravenous drugs user, public awareness, education and improving health care facilities is urgently needed.

Alizadeh et al. (2010) analyzed that worldwide HDV infection is although decreasing but the consequences of HDV infection can never be denied. The main objective of their study was to estimating HBV co-infection with HDV and correlate different factor of infected individuals. They perform their study on those patients who were infected chronically with hepatitis HBV in Hepatitis Community Center since form 2002-2007 at Hamedan Province. They have completed a valid questionnaire which comprising demographic information and past history of hepatic disease for each individuals. After performing primary test they have assayed enzyme-linked immunosorbent assay for confirmation and measuring antibodies to HDV. They found that IgG antibodies against HDV were positive in 14 (17.3%) subjects in a total of 81 HBsAg positive patients they have screened. Similarly only one anti-HDV IgM positive

patient they reported in this study. They found that two (14.3%) of the anti-HDV IgG positive patients were women. Similarly they reported that a total of 24 (35.8%) women were negative for IgG to anti-HDV (p = 0.21), which were examined in this study, and of these, 6 (42.8%) were hepatitis e antigen positive while 17 (25.4%) of the anti-HDV IgG negative female were positive for hepatitis e antigen (p = 0.16). They found that anti-HDV IgG confirmed positive was 28.6% and 39.2% of the cases were negative for anti-HDV IgG (p = 0.31) among these chronic hepatitis B infected patients. Finally they concluded that it is necessary for the policy maker's and healthcare personnel to determine the risk behavior which associated with hepatitis B and hepatitis D viral co-infection as well as find out the reasons for this increased burden of HBV and anti-HDV co-infection.

Ali et al. (2011) estimated that hepatitis B carrier rate is round about 3-5% and about 7 to 9 million hepatitis B virus carrier are found in Pakistan. They included all the available literature and studies performed in Pakistan which were focused on the, awareness status, risk behavior, prevalence and genotypes of the HBV in this article. They collect all these published data since from 1998 to 2010 from DOAJ, PubMed, Google Scholar and PakMediNet and found one hundred and six different studies which were done on Pakistani population. They analyzed and determined each group mean and standard deviation. They estimated that 4.3318% ± 1.644%, of hepatitis B virus infection prevails in common population, similarly they also reported that (3.93 % ± 1.58 %) percentage of healthy people who donating blood were infected, personal who recruits in military were (4.276 % ± 1.646 %), liver diseases individuals (27.54 % ± 6.385 %), prisoners (5.75% ± 0.212%), HCC patients (22 % ± 2.645 %), multiple transfused patients (6.223% ± 2.121%), patients with history of surgery (7.397 % ± 2.012 %), patients abnormality of liver cirrhosis (28.87 % ± 11.90 %), persons who take care of patients (3.25 % ± 1.202 %), patients with hepatitis (15.896 % ± 14.824 %), pregnant female (5.872 % ± 4.984 %), ophthalmic disease patients (3.89% ± 1.004%) and users of illegal injection of drugs (14.95 % ± 10.536 %). They found that the most common prevalent genotype in the population of Pakistan among all HBV infected individuals was genotype D (63.71%). They finally concluded that strict strategy of awareness programs

and Mass vaccination should be the practiced on immediately basis especially in those populations win which the infection rate of HBV is more than 5%.

Attaullah et al. (2011) demonstrated that the complication, treatment, possibly vaccination and severity of HBV infection are due to the difference in the genotype structure and function of the hepatitis B virus. They find out that in past no such work done on the prevalence of HBV genotyping in Afghanistan, that clearly explain the true picture of the more prevalent genotype. So they performed their study on HBsAg positive patients and a total of 214 Surface antigen positive individuals were enrolled in this work. They also performed screening of all the individual with anti-HCV antibodies, anti-HIV antibodies and all of the patient they were selected were negative for both anti- HIV and anti-HCV. They confirmed HBV DNA with nested PCR and finally processed all of the HBV DNA positive samples for confirmation of genotyping (A-F). They reported that a total of 168 (78.5 %) samples were men and 46 (21.49 %) of them were women, and they found that all of the confirm cases were fall in the ages group of 18 to 71 years. They testify that the more prevalent genotype existed was genotype D (35.67%) in Afghani's population. They also notify that infection with C genotype was 32.16 % subsequently A genotype was (19.30 %), and B genotype (7.02 %) although 6.07 % of the infected patients were not typed. They analyzed that this study clarify that various, multifarious genotype distribution is present among the population of this region. They finally concluded that a large scale work on the prevalence and genotyping is the need of time, that finds out geographical, genetic divergence and characteristics of the virus in the country.

Awan et al. (2012) demonstrated that the analysis of HBV genotype is very important due to the epidemiological point of view, but there is no such capacious amount of data accessible for HBV genotypes that were established currently and the mixed infection of more than one HBV genotype is very limitedly known. Their study was aiming to analyzed the genetic and molecular level characterization of hepatitis B virus genotypes in those patients who were already surface antigen of HBV positive in the local community of Khyber Pakhtunkhwa Pakistan. They randomly enrolled a total of

713 HBsAg positive cases in their study. They processed all of the HBV DNA positive cases which were confirmed by nested polymerase chain reaction for HBV genotyping with type specific primers. They reported that genotype A (33.66%) was the more prevalent genotype followed by genotype D (29.5%), genotype F (1.40%), genotype C (2.10%), and similarly they also found mixed genotypes A+D (10.52%) although 5.9% of the individuals were untypable. They did not reported any case of genotype B and E in this study. They concluded that genotype A is present in high ratio among HBsAg positive individual of KPK and different HBV genotype is distributed in this region. They further concluded that in this area more research work needed that find out the molecular, geographical characteristic and genotyping of hepatitis B virus.

Badar1 *et al.* (2012) arranged a cross-sectional study to estimate the hepatitis B virus genotype prevalence of in Pakistani population. They investigate that HBV which is one of the most dominant causative agent of chronic and acute liver illness throughout the world with serious morbidity and mortality. They noticed that HBV have high genetic variation which result in the expression of eight different genotypes (A to H), and each one of them have different geographical distribution. They also found that various genotype of HBV have differently circulating among different community population globally indicated the pattern of human migration. They perform this prevalence cross-sectional study to analyzed the most predominant genotype in Pakistani population and a total of 255 HBV ELISA confirm positive cases were tested. They include different HBV patients who were visited to the local hospitals in Pakistan at Faisalabad, Lahore and Islamabad. They found that a total of 214 samples were PCR positive and hepatitis B viral DNA were not detected in the remaining 41 samples. They used regular PCR in first and nested for second round PCR to amplify the S-gene of HBV PCR positive samples. They used Restriction Fragments Length Polymorphism to digest 2nd round PCR products. They used five different restriction enzyme (*Hph*I, *Nci*I, *Alw*I, *Ear*I *and Nla*IV) that determined the genotype-specific sequences. They identified that only two genotypes were clearly prevalent among all of the selected positive individuals which were genotype C and D. they found that only 21 cases (9.81%) of them were genotype C and remaining 195 (91.1%) of them genotype D in the local community of this region. They

also concluded that the algorithm chose in this study that can be used to analyzed different HBV genotypes.

Chakraborty et al. (2005) concluded that hepatitis D viral infection rate reach to lower level in some of the geographical region in the world. They found that no such work has been established that clarify this kind of change in HDV infection rate in India like those occurring in other regions of the world. They initiated this research work to estimate the seroprevalence of HDV co-infection with HBV related liver illness. They include mostly those patients who come to the New Delhi Government hospital, and to determine any modification in its epidemiology by correlation of their data with previously reported seroprevalence results. They found that a total of one hundred twenty three patients with hepatitis B virus related liver infection containing 32 confirm subjects of acute viral hepatitis, five of them were suffered from fulminant hepatic failure (FHF), 37 of them have been chronically infected with HBV(CH), 46 individuals have liver cirrhosis and only 3 of them have reported with hepatocellular carcinoma (HCC).they screened all the patients with anti-HDV by using ELISA kits. They demarcate both convalescent and active HDV infection by performing anti-delta IgM and IgG anti-delta test. They found that male and female ratio of the patients of the current study was 11:5 and the mean age of the individuals was 35.6 +/- 3.3 yr. They found that 13 (10.6%) individuals were confirmed with hepatitis D viral infection out of a total 123 subjects they were screened, 9 (7.3%) cases were previously infected (Immunoglobin G positive, Immunoglobin M negative) and the remaining 4 (3.3 %) were recently infected (IgM anti-HDV positive). They also classified that evidence of hepatitis D virus infection in chronic hepatitis, acute viral hepatitis, cirrhosis, fulminant hepatitis, and hepatocellular carcinoma groups was found in 15.2, 3.1, 8.1, 20, and 33.3 patients, respectively. They concluded that our report indicates that HBV co-infection with HDV is not very prevalent in Indian population. They also concluded that hepatitis D virus epidemiology in this region of the world changing towards decline prevalence.

Cho et al. (2012) figure out the prevalence and risk factors for surface antigen of HBV positivity in pregnant Ghanaian women. They studied a total of fifteen hundred

pregnant female in Eastern part of Ghana. Well qualified nurses complete the task of obtaining patients history and other related valuable data during interview by using authentic questionnaires. They screened all of these pregnant women for HBV, HIV, sickle cell anemia and hemoglobin level which were randomly used for antenatal examination. They reported that HBsAg positivity rate among pregnant women were 10.6%, they also analyzed that prevalence rate were different among district, in Kwahu West was (13.8%), in Upper Manya was (12.4%) and in Yilo Krobo was 2.2% respectively. They reported that in women with depression HBsAg positivity was relatively higher (odds ratio, 3.74; 95 % confidence interval, 2.13 - 6.57) and Human Immunodeficiency virus (odds ratio, 2.03; 95% CI, 1.06 - 3.89). They analyzed that some of the factor like education, gravidity and age were not related to HBsAg positivity. They noticed that in newborn of HBV-positive mothers immunoglobulin against hepatitis B was not administered at birth in public health facilities in Ghana. They also noticed that during 6 weeks of age vaccination against hepatitis B viral infection was administered as part of a regular vaccination agenda. They concluded that HBsAg screening of pregnant female and vaccination of newborn after birth is recommended to prevent HBV infection in this region.

Ghadir et al.(2012) concluded that in acute or chronic HBV infected patients hepatitis D virus (HDV) viral co-infection or super infection may also found because HDV is a defective RNA virus that do not cause infection alone and depend on the surface antigen (HBsAg) of HBV for its replication. They noticed that in Iran very negligible data available regarding the routes of HDV transmission. They further summarize different risk factors involved in HDV infected population of Iran, including traditional phlebotomy, family history, tattooing, bloodtransfusion, endoscopy, dental interventions, surgery and war injury. They included a total of 3690 samples randomly from different region including 7 rural clusters and 116 urban clusters. They have screened all the individuals with HBs antigen and all of the positive HBV cases were processed for anti-HDV. Only 48 subjects (1.3%) were found positive for hepatitis B infection and only one of HBsAg positive case had HDV infection. They found that the prevalence of HDV was 0.03% in Qom Province. They also found that HDV prevalence

in HBsAg positive subjects was 2%. They noticed that the only case which was anti-HDV positive had a history of dental surgery, tattooing and surgery. They concluded that there is no such symbolic There was no significant association between HDV infection surgery history, or dental surgery and tattooing. They found that in Qom province the prevalence of hepatitis D is the lowest similar to a study in Babol (north of Iran).

Gomes et al. (2006) assessed that hemodialysis individuals are at major risk for hepatitis B virus infection. They organized analysis of hemodialysis individuals of the Goiás state of Central Brazil, focus of their study was to determine the hepatitis B viral infection prevalence, to measured the risk factors, and to cross-examined distribution of HBV genotypes. They investigated a total of 1095 subjects in their study in fifteen dialysis wards. They used ELISA assay for all of the serum samples to determined serological markers of HBV. They have tested all of the surface antigen positive for HBV DNA amplification by PCR and HBV genotype was confirmed by restriction fragment length polymorphism. They found that Global Hepatitis B viral infection prevalence was 29.8 % (95 % CI: 27.1-32.5). They noticed that HBV positivity was associated with different risk factors and demonstrated that male gender, time duration on hemodialysis, and transfusion of blood before 1993 were identified. They found that DNA of HBV was observed in 65.4 % of the HBV surface antigen positive cases. They examined thirteen of seventeen HBV DNA positive cases for genotyping. They noticed that genotype D (61.5%) was found to be the most prevalent, subsequently genotype A was found in (30.8 %), while F genotype was identified in only 1 (7.7 %) individual.

Gutiérrez-García et al. (2011) concluded that individual that negative for surface antigen of HBV but have a detectable amount of DNA of HBV in the liver tissue of infected person is known as occult hepatitis B virus infection (OBI). They noticed that worldwide OBI prevalence is quite different depending on the level of endemic disease in various regions of the global world, the different techniques used in the studies, and the different populations studied. They studied that many research work have been done in different geographical regions and in different categories of population for OBI prevalence. They concluded that all these studies show that OBI prevalence rate assume

to be much increase among individuals at great risk for B viral infection and with liver illness than among those subjects who were at lesser risk of infection and apart from liver illness.

Helmy et al. (2006) evaluated the prevalence of lonely anti-HBcore in patients which were infected chronically with hepatitis C virus, and correlate it with severity of disease. They screened all HCV infected bring up to King Faisal Specialist Hospital for anti-HBs, surface antigen of hepatitis B, and anti-HBc. They included about 169 cases in their study who were both negative for B surface antigen and anti-HBs. They noticed that 59 patients had cirrhosis which was confirmed by biopsy, and one hundred and ten had chronic active hepatitis. They found that 85 (50.3%) of patients have positive results for anti-HBc out of these 169 patients. They distinguished that CAH patients had quite greater prevalence of isolated anti-HBc than those subjects which had cirrhosis, which were 71 (64.5 %) and fourteen (23.7 %) respectively ($P < 0.001$). They measured HBV DNA in twenty-five patients by using qualitative PCR. They found that PCR positive results were observed in 3 of them (12 %; OBI. They analyzed that isolated anti-HBcore alone is frequent in Saudi patients which have chronic hepatitis C infection, while isolated anti-HBc is relatively frequently found in those with CAH than those with cirrhosis. They concluded that potentially infectious individuals with isolated anti-HBc were missed if a screening strategy that were only focus on testing the patients only with HBsAg and anti-HBs.

Hassan et al. (2011) concluded that hepatocellular carcinoma, liver cirrhosis and fibrosis is developed due to the results of occult HBV infection. The objective of their research work was to analyzed the occult HBV-genotypes in HCC patients. They collected tissue and serum samples from a total of forty HCC patients. They designed 3 sets of primers to analyzed HBV-DNA amplification by nested-PCR, these primers collectively cover the genome of hepatitis B; Core, X genes and Surface. All of the PCR positive samples were further tested for genotyping by using type-specific primers. They found that 62.5% patients were positive for intrahepatic occult HBV-DNA, whereas 22.5% of HCC patients were detected with serum occult HBV-DNA. They found that

about 10% of both anti-HBcore and anti-HBs, positive patients had occult hepatitis B virus in serum. They also noticed that 63 % had intrahepatic DNA of HBV, and 21 % serum samples had HBV-DNA in those patients who were serologically negative for HCV. They further found the genotyping distribution of all positive samples which were (32%) had D genotype of HBV and B genotype was found in (24 %) predominantly account for intrahepatic HBV infections in hepatocellular carcinoma individuals, but A genotype (4 %) and genotype C (8%) infections were the rarely recognized. They concluded that this was the first work perform on the genotypes of occult HBV infection in hepatocellular carcinoma individuals. They finally suggested that the development of HCC may be the results of genotype D or B which may influence the aftermath of HBV infection.

Katsanos et al. (2004) analyzed that in 1991, due to the socioeconomic crisis and political disturbance in Albania, huge population were migrated to in the form of refugees to Apulia region of Southern Italy and to the Northwestern Greece (Ioannina region). They founded that a high endemicity of HBV infection were present among these Albanian refugees and the Seroepidemiological data was collected on the basis of different hepatitis viral serological marker distribution. More recent and authentic epidemiological enlightenment about hepatitis B virus marker prevalence especially of the growing (young), non-vaccinated Albanian community became accessible through a mutually study done by the national Greek-Albanian, on prevention hepatocellular carcinoma and viral hepatitis (known with the composition as the HEPAGA study). They find out that among all of the tested Albanian refugees HBsAg(+) was measured in 11.89% groups of individuals whereas immunoprotected against HBV group was found in only 21.19%. An important task for all countries to gain or fail to gain the WHO 1997 goals because every country was committed politically and socially to vaccines and preventive medicine, they all were also given a target to educate and acknowledge common people and medical community. They noticed that in EastSouthern Europe, where chronic and acute hepatitis B infection was a main health hurdle. Policy of vaccination to selected group (risk group) did not produce positive outcomes of this infection and HBV transmission from growing individuals, carrier pool was not able to

stop. They analyzed that massive number of migrated people from high to moderate or less endemici areas such as Italy and Greece result in a new dynamic epidemiology of hepatitis B transmission. They also noticed that continuing programmes of vaccination against hepatitis B infection is one of the critical obstacle for the near future. They concluded that different challenges which were found in this area were, enhancing coverage vaccine, ongoing support in order to fulfill a continuous delivery of vaccine, other facilities related to them and finally to successfully evaluate the efficacy of currently implemented vaccination programmes.

Khan et al. (2011) suggested that hepatitis B infection is critical public health problem in the Pakistani population. They also found that B viral hepatitis is the main cause of cirrhosis, chronic hepatitis, hepatocellular carcinoma and fibrosis. They noticed that in region of low economic status increase frequency of HBV infections has been found. Due to poor hygienic measure and lack of interest of government authorities the epidemiological picture remains high and no major change has been documented in spite of effective vaccination strategy especially in rural region of Pakistan ("67.5 % of the total public). They performed this study within internally displaced persons of Malakand Division of Northern Pakistan which were migrated due to war against terrorism and their main objective was to estimate the risk factors and prevalence of HBV infection. They collected blood samples of about 950 IDPs who were suspected for HBV infection (including both women and men) and all of these samples were assayed by commercially produced ELISA kits for HBeAg, B virus surface antigen, Anti HBe antibodies and Anti HBs. They processed all of the positive samples for detection of DNA of HBV by real time PCR analysis. They found that a total of 21.05 % of individuals were confirmed DNA of HBV positive among them 21.5% were women and 78.5% were men. They analyzed that majority of the HBV positive patients were from the Malakand and Dir (lower) district areas. They found that increase-risk of disease was observed in the older individuals 29.13 % (46 to 60 years), while a rare rate of infection (11.97 %) was noticed in patients aged <15 years. They noticed that most commonly observed risk factor in all these individuals were, sharing of blades of razor, needles used for tattooing and syringes, sexual activities, socioecomic conditions, and lack of awareness during the cohort of

patients. They concluded that this show increase prevalence of HBV infection for the first time in the rural regions of Northern Pakistan. They also observed that due to increase exposure to the general risk factor high prevalence were noticed in gender- and age-dependent. They finally concluded that expended immunization strategy possibly awareness about risk factor are necessary for the prevention and transmission of hepatitis B infection.

Khan *et al.* (2011) suggested that many risk factors are determined which aftermath the spread of this virulent disease. They analyzed the prevalence of hepatitis B viral infection during 2008 to 2010 in those individuals which were suspected, in Punjab province of Pakistan. Their study was mainly focused to measure the risk factor and the epidemiology of HBV infection. They used Real Time qualitative PCR to confirm the disease status and to detect HBV DNA in a total of four thousand eight hundred and ninety patients who were infected with chronic liver disease. They used specially designed questionnaire for taking history of the suspected individuals and their questionnaire cover all the information and possible risk factor for HBV infection. They tested a total of 4890 positive cases which were confirmed by ELISA and were assayed for viral Hepatitis B infection. They found that among all the individuals they screened about 3143 were positive with B hepatitis, including 68.15 % men and 31.85 % women. They noticed that males were predominantly infected than females with a positivity ratio range of 2.14: 1. They found that during the period of three years the frequency of infection was increase with interval of time. They found that majority of the infected people were fall within the age group of 21 to 30 which was 34.93 % and simultaneously 23.83 % in age group of 31-40. They further noticed that only (13.39%) were those patients whose ages were between11-20 year. They found that infection rate of patients was declines with high age as observed by groups ages between41 to 50 (16.13 %) and among ages between 51 -60 (7.09 %). They also found that infection rate seem to be decline in children 1.49 % of age group 0-10 and very old age group of more than 60 years were noted 1.65 %. They observed that analysis on the basis of risk factor shows that different common risk factors contributing the transmission of HBV include 26.60 % reused syringes, barber risk (23.60 %), History of injection 26.19%, dental risk (11.20%)

blood transfusion (4.04%), and procedure of surgery (4.26 %). They suggested that among all these practice and trend of personal objects sharing was very common. They further noticed that transmission of HBV infection was mainly due to major risk factor like dental procedure, barber risk, history of injection, surgery and reuse of syringes. They pointed out that exposure rate to common risk factor of male were greater as compared to female. They also analyzed that children and old age group people were less frequently infected as compare to younger age group. They found that history of injection, risk of barber and syringes reuse' were observed the highlighting risk of transmission of HBV infection. They concluded that priority steps should need to taken by Government official to lower HBV transmission rate and to minimize the burden of B viral hepatitis from the Punjab province of Pakistan extend awareness program and vaccination strategy should be implemented on the urgent basis.

Khan et al. (2011) described that HDV causes super infection or co-infection with serious complication as compared to isolated hepatitis B viral infection. They analyzed that HDV depend and propagate in the host which is previously infected with HBV because hepatitis delta virus is sub satellite virus. They claimed that no such work has been done on the molecular level in this region so their aim was to investigate the molecular epidemiology of hepatitis Delta virus (such as a co-infection) in various geographical areas of Pakistan. So for this purpose they collected a total of 228 B surface antigen confirmed samples from various geographical areas of the country. They tested HDV RNA in those subjects which were positive for HBV DNA PCR. They amplified B viral DNA and RNA of HDV by utilizing reverse transcriptase polymerase chain reaction, real-time PCR and nested PCR. They detected HBV DNA in 190 (83.3%) samples out of total two hundred and twenty eight surface antigen confirmed sera from different patients. They found out HDV RNA in 53 (28%) patients out of 190 subjects which were confirmed HBV DNA positive. They further classified these 53 HDV positive cases into male, 37 (69.8%) and female which were 16 (30.2%) respectively. They pointed out that male patients were predominantly infected ($p < 0.05$) as compared to female patients. They observed that mostly patients 41 (26.8%) were positive and their ages were below 40 years as compare to the patients whose ages were above 40 years

were 13 (31.7%). They also found that at provincial level the prevalence of Delta hepatitis was 67 %, 6 % and 4% in Sindh, Khyber Pakhtoonkhaw and Punjab respectively. They concluded that HDV infection can't be ignored in Pakistan because its infection frequency is much higher in the Province of Sindh ($p < 0.01$) and six male ($p < 0.05$).

Kim et al. (2011) demonstrated that hepatitis D viral infection is predisposal factor of more serious acute and chronic liver illness in individuals already infected with B viral hepatitis. They analyzed that although worldwide HDV infection significantly decreases but in some countries of the world it is still major health issue. They performed this study to measure the infection frequency and to investigate clinical features of Delta hepatitis co-infection in those subjects which were chronically infected with hepatitis B virus in highly endemic region of Korea. They included a total of nine hundred forty chronically infected HBV individuals with in age's range of 18-94 years (median age, 48) and among them men were, 64.5%. They processed all of the B surface antigen confirmed cases with a positive history of at least half year HDV antibodies screening. They utilized sera of those cases that were positive for anti hepatitis D antibodies for phylogenitic analysis, molecular analysis, sequencing and amplification of small portion of the HDV delta antigen. They evaluated virological marker and clinical features. They noticed that chronic hepatitis B infection was 44.7%, inactive HBsAg carriers was 29.5%, hepatocellular carcinoma was 8.0% and cirrhosis was accounted for 17.9% respectively. They found that anti-HDV positive rate was 0.32%, because only 3 subjects were observed positive for antibody to HDV. They noticed that all three cases who were positive for antibody to HDV were inactive surface antigen carriers. They found that only two patients HDV RNA were confirmed positive for PCR. They further verified and confirmed Phylogenetic analysis which provide results that both RNA positive patients carried Delta virus genotype 1. They finally concluded 0.32% prevalence of HDV was noticed in Korea which was very low. They analyzed that this data showed that only HDV genotype 1 was noticed and detected in inactive HBsAg carriers. They suggested that in chronic HBV infection, HDV co-infection may have not a serious clinical impact in Korean individuals.

Riaz et al. (2011) investigated that hepatitis viral infection is one of the critical health problems all over the world, specifically in Asian (South East) countries among them Pakistan is also including in this category. In this part of the world B viral hepatitis and hepatitis C viral infections are found to be increasingly endemic. Hepatitis delta viral infection is also not be neglected throughout the world. Viral hepatitis C, HBV, and Delta virus use similar mode transmission which may becomes the consequences triple or dual viral infection that can occur simultaneously at the same time in a proportion of patients. Development of cirrhosis of liver and carcinoma of hepatic cells is the result of HBV and HCV. Chronic Delta hepatitis is also one of the factor for development of damage to liver tissue with oncogenic potential beside cirrhosis of liver and HCC. They reported all the available literature in this review article including pathogenesis, epidemiology, replication, symptoms, treatment, diagnosis, preventive measure and disease outcome of triple hepatitis from PakMediNet, DOAJ, PubMed and Google scholar. They include about seventy four various published work from 1983 to 2010. In this article they particularly review triple hepatitis, HBV, HDV and HCV.

Shaikh *et al.* (2012) conducted a study which find out rate of triple hepatitis in B surface antigen confirm individuals and analyzed the outcome of different parameter like radiological and LFT results. They include seventeen hundred and thirteen B surface antigen positive cases from Jan. 2008 till June 2011 of Chandka Medical College Hepatology clinic, Larkana. They collect patients history and with drawl sample of blood for determination ofLFT, HCV antibodies, Delta virus antibodies, amplification of B viral DNA, RNA of hepatitis D and HCV. They observed Ultrasonic feature of hepatitis. They analyzed triple hepatitis confirmed and negative individuals and compare their radiological feature, biochemical investigation and serological profile. They used SPSS v 19 for numerical analysis. They reported that out of seventeen hundred and thirteen cases, B viral hepatitis DNA was amplified in 308 (18 %), RNA of Delta virus in 896 (52.3 %), while RNA of HCV in 148 (8.6 %), antibodies to HDV present 1116 (65.1 %) and antibodies to HCV was present in 420 (24.5 %). They found that triple hepatitis (hepatitis D+C+B) was determined in two hundred sixty eight (15.6 %) cases. Men ($p<0.000$) cases

among TH confirmed patients were 220 (82.1%). After examined Ultrasongical feature of TH, chronic liver disease of advance stages was observed. In majority of cases parameter of LFT were elevated significantly. They finally concluded that high frequency 15.6 % of triple hepatitis was found in B surface antigen positive subjects. They also concluded that men were more affected and liver disease of advance stages was observed in infected individuals.

3.1 Materials and methods

3.1.1 Material and Equipment:

- Micro plate reader
- Automatic micro plate washer with Incubator
- Centrifuge machine
- Microcentrifuge for PCR
- Vortex
- Thermocycler
- Disposable powder-free gloves
- Pipette 10ul – 100 µl
- Pipette 100ul – 1000 µl
- 20µl and 200µl adjustable pipettes and aerosol barrier tips
- Sterile, nuclease free 1.5 ml microcentrifuge tubes
- Laboratory marking pen
- MBS ITALY ELISA kit
- DNA Extraction kit of Saceae
- Agarose Gel
- 1X TBE buffer
- Electrophoresis Tank
- UV transilluminator for band detection
- Purified water/ Distilled water and deionized water (RNase and DNase free)
- Disposable Pipette tips (yellow & blue)
- A lid for polystyrene 96-well plates or a protective film for plates
- Tissue paper
- Gloves
- Lab coat
- Stop watch
- Waste container

3.2.1 Design and settings

This part of this cross-sectional study was performed from 2011 to 2013 in KPK Province at sentinel Site HMC Peshawar except from molecular biology work including PCR, Gel electrophoresis and writing of this work was performed at Department of Microbiology QAU Islamabad. .

3.2.2 Explanation about the area assigned for study

Pakistan has five provinces, FATA and a capital territory. The Khyber Pakhtunkhwa is one of the five provinces of Pakistan. West and South extremities of KPK are attached to the FATA. The South-East border is joined to Punjab, and the North-East border connected to Gilgit-Baltistan and the East border of KPK are merged with Azad Kashmir. KPK South border are connected to the Baluchistan province while its North-west borders are cognate with Afghanistan. The majority of the population of the KPK is Pashto speaking known as Pathan, and small category of other traditional group also living in this region. The most dominant speaking language of KPK is Pashto and the historical city Peshawar is the capital of this province. KPK consist of a total of 24 districts.

3.3.1 Sample Collection:

Aseptic technique of safe blood samples collection was followed during phlebotomy. Samples of blood were collected from the patients of Medical and Gastro OPD of Hayatabad Medical Complex Peshawar after performing LFTs. The samples were collected from those patients who LFTs were elevated and were fall within the specific case selection criteria. A total of 3 to 5 ml sample of whole blood was collected in a jell tube (vacutainer) from each person selected for the study. After centrifugation serum was separated and stored at -20°C for further testing. Standard operating procedure were strictly followed for each single steps to reduce the errors.

3.3.2 Data collection

The study was conducted in Hayatabd Medical Complex Peshawar and data ollection was performed from 2011 to 2013. A specially designed questionnaire was established for collection of patient data which was completed by a trained interviewer, containing information regarding age of the patient, sex, socioeconomic status, vaccination history, demographic, anthropometric data and various risk behavior for the study objective.

3.3.3 Ethical approval

Written or informed consents were obtained from all participants before data collection.

3.4 Biochemical Test

The liver function tests accomplished included serum glutamic-pyruvic transaminase (SGPT), bilirubin and alkaline phosphatase (ALP) by using Cobas C111 of Rosh and followed manufacturer's instructions and SOPs.

3.4.1 ELISA

All the patients with elevated liver function test were processed to screening for HBsAg, HBcore total, Anti HDV, HBcore IgM and Anti-HCV by using 3rd generation ELISA according to manufacturer's instructions.

3.4.1.1 ELISA procedure for HBsAg and Anti-HBcore Detection:

- All the reagents/kit was brought to room temperature at least one hour before use. All the samples which were to be tested were thawed.
- A1 well was leaving empty for blank. 50 ul of negative control was dispensed in next three wells (A2, A3, A4), then 50 ul positive control and 50 ul of sample was added into their respective wells.
- A total of 50 ul of Conjugate was added into all wells except A1. The content of the wells was mixed by careful tapping on the edge of the plate. The plate

was covered by a plate lid and the reaction mixture was incubated for 60 minutes in microplate incubator at 37^{O}C.

- The plate lid was removed and plate wells were washed for 5 times with working washing solution in Microplate washer.
- A total of 50 ul of substrate A and 50 ul of substrate B were transfered into all the wells along with A1. The plate was incubated for 15 minutes at 37 ^{O}C in microplate incubator and the lid of incubator was covered to avoid light.
- Finally 50 ul of stopping reagent was added into all wells. For measuring microplate background the Blanked was read at 620-630 nm. Then the absorbance of samples and controls was read at 450 nm in microplate reader.

3.4.1.2 ELISA assay scheme for HBsAg and Anti-Hbcore

Reagents	**Blank**	**Control**	**Samples**
Controls	-	50 ul	-
Samples	-	-	50 ul
Conjugate	-	50 ul	50 ul
• Plates were covered and incubated for 30 minutes at 37 ^{O}C • After 30 min cover was removed and were washed 5 times with working washing solution in microplate washer			
Substrate A	50 µl	50 µl	50 µl
Substrate B	50 µl	50 µl	50 µl
Then all the samples were incubated for 10 minutes at 37 ^{O}C in microplate incubator and the plates were covered by the lid of incubator to avoid light			
Stoping solution	50 ul	50 ul	50 ul
For measuring microplate background the Blanked was read at 620-630 nm. Then the absorbance of samples and controls was read at 450 nm in microplate reader.			

3.4.1.3 Procedure for Anti-HDV and Anti HCV:

- All the reagents/kit was brought to room temperature at least one hour before use. All the samples which were to be tested were thawed. .
- A total of 100 µl of sample diluent was dispensed to all wells except A1 which was leaved empty for blanking. 10 µl of negative control was dispensed in next three wells (A2, A3, A4), then 10 µl positive control and 10 µl of sample was added into their respective wells.
- The content of the wells was mixed by careful tapping on the edge of the plate. The plate was covered by a plate lid and the reaction mixture was incubated for 60 minutes in microplate incubator at $37^{O}C$.
- The plate lid was removed and plate wells were washed for 5 times with working washing solution in Microplate washer.
- A total of 100 µl of Conjugate was added into all wells except A1. The plate was covered by a plate lid and the reaction mixture was incubated for 20 minutes in microplate incubator at $37^{O}C$.
- The plate lid was removed and plate wells were washed for 5 times with working washing solution in Microplate washer.
- A total of 50 ul of substrate A and 50 ul of substrate B were transfered into all the wells along with A1 The plate was incubated for at least 10 minutes at 37 ^{O}C in microplate incubator and the lid of incubator was covered to avoid light.
- Finally 50 µl of stopping reagent was added into all wells. For measuring microplate background the Blanked was read at 620-630 nm. Then the absorbance of samples and controls was read at 450 nm in microplate reader.

3.4.1.4 ELISA assay scheme for Anti-HDV and Anti-HCV

Reagents	Blank	Control	Samples
Sample diluents	-	-	100 ul
Controls	-	10 µl	-
Samples	-	-	10 µl
• Plates were covered and incubated for 30 min at 37 ^{O}C • After 30 min cover was removed and were washed 5 times with working washing solution in microplate washer			
Conjugate	-	100 µl	100 µl
• Plates were covered and incubated for 20 min at 37 ^{O}C • After 20 min cover was removed and were washed 5 times with working washing solution in microplate washer			
Substrate A	50 ul	50 ul	50 ul
Substrate B	50 ul	50 ul	50 ul
Then all the samples were incubated for 10 minutes at 37 ^{O}C in microplate incubator and the plates were covered by the lid of incubator to avoid light			
Stoping solution	50 ul	50 ul	50 ul
For measuring microplate background the Blanked was read at 620-630 nm. Then the absorbance of samples and controls was read at 450 nm in microplate reader.			

3.4.2 Extraction of DNA Procedure:

A total of 150 ul of HBsAg positive serum was used to performed DNA extraction by using "**Saceae**" Biotechnologies extraction kit according to manufacturer's instructions.

1) A total of 600 µl of prepared Buffer RAV1 (RAV1+RNA carrier) and 20µl of proteinase K was added to150ul of serum sample in a 2 ml microcentrifuge tube.

The contents of tube was mixed by pulse vortexing for 20 seconds and the mixture was then incubated at 70 ^{O}C for 5 min.

2) Then 600 µl of absolute ethanol was also added and mixed by pulse –vertexing for 15 sec. A total of 700µl of the above mixture was shifted to spin column (Ribo virus column), and was Spined at 8000 x g for 60 second.
The above step was repeated with the remaining mixture (new collection tube).

3) The filtrate tube was dropped then the column was planted in a new accumulating tube and a total of 500 ul RAW buffer was transferred to it and then spined at 8000 x g (8000rpm) for 1 minute.

4) After spinning, the accumulation tube was dropped; the column was shifted in new tube for collection. A total of 600 µl RAV3 buffer was transferred to the column and then spined at 8000 x g for 1 minute.

5) After spinning, the filtrate was dropped and the empty column was planted in 2 ml tube for collection of sample mixture. A total of 200 µl of RAV3 buffer was transferred to the column and then spined at 11000 x g for five minutes to clear away ethanolic buffer RAV3 completely.

6) The column with open cap was incubated at 70 ^{O}C for 1 minute to clear any remaining traces of ethanol.

7) Then column was shifted in a new, sterile 2.0 ml centrifuge tube. Finally 50 ul RE Buffer (preheated to 70 ^{O}C) was transferred and then incubated for 1-2 minutes. Then centrifugation was done for 60 seconds at 11000 x g to collect the DNA.

8) The DNA was Stored at -70 ^{O}C.

3.4.2.1 DNA amplification and detection

The DNA was amplified by using the process of PCR. Two set of primers were used for the amplification of DNA, these were FA1-L and FA1-R, the sequences of the primers were as, forward primer TTTCACCTCTGCCTAATCATCTC, and the sequence of the reverse primer was TCTTGTTCCAAGAATATGGTG. Similarly the second pair of primer sequences was as under, forward primer, FA3-L, CTGCTGGTGGCTCCAGTT and the sequence of the reverse primer was as FA3-R GCCTTGTAAGTTGGCGAGAA. PCR mixture was prepared by taking 4ul of master mix (Commercially available), 0.6ul of forward primer, 0.6ul of reverse primer, 9.8ul of PCR water and 5ul extracted DNA for each sample to make final volume of 20 ul. The PCR conditions were optimize as, initially denaturation at 95 ^{O}C for two minutes 30 seconds, which were followed by subsequent 35 cycles of denaturation at 94^{O}C for one minute, the annealing temperature was set at 55 ^{O}C for one minute and extension of amplified sequence at 72^{O}C for at least two minutes, finally extension at 72^{O}C for ten minutes.

PCR Mixture for each sample	
PCR Master Mix	N x 4 ul
DNA	N x 5 ul
Forward primer	N x 0.6 ul
Reverse primer	N x 0.6 ul
Nuclease free water	N x 9.8 µl
Total volume	N x 20.0 µl

After the process of PCR amplification the PCR results were scrutinized on Agarose Gel Electrophoresis. 1% of Agarose Gel was adjusted by weighing 0.4 gram of agarose which was dissolved in 40 ml of 1X TBE buffer. The buffer was prepared in deionized water. The gel was prepared and 2ul of ethidium bromide was added to the gel and was let for solidification. After solidification the gel was put in the gel tank containing 1X TBE buffer. Samples were loaded by mixing the 5ul of the samples with 1ul of loading dye and were loaded to the wells of the gel. All the samples were run in the gel for 45. Voltage applied was 100V and the current was 400milli amperes. After completion of the electrophoresis the gel was evaluated under UV transilluminator for the presence of the bands of DNA. The picture of gel was taken with gel documentation equipment and the band of 1000bp size was observed. The specific HBV DNA product was demonstrated by correlate it with 100 bp DNA ladder, used as DNA size marker.

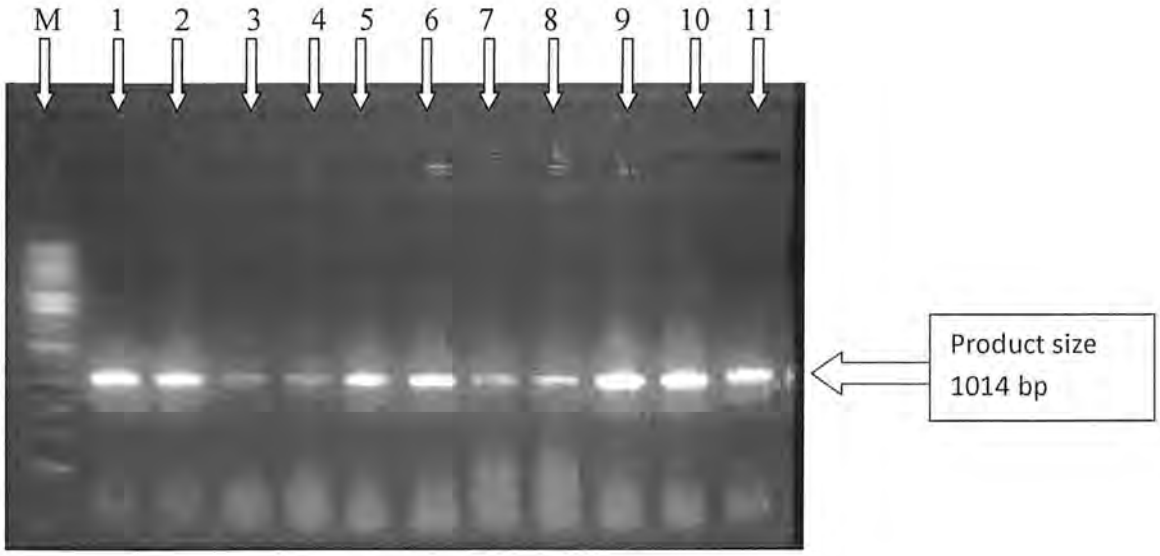

Figure 01. Agarose Gel Electrophoresis image.

This image shows the PCR amplified product of HBV DNA. The size of the amplicon was 1014bp. M shows the DNA marker of 1kb size. Lane 1-11 indicate the PCR product of different samples.

3.5 Analysis of Data

The numerical examination of research data was achieved by using SPSS software.s

RESULTS

In total, 212 HBsAg positive individuals (confirm by ELISA) consisting of 114 male and 98 female who fulfilled the criteria of study were included for analysis. Higher percentage of male patients is found to be infected as compared to female. All of the HBsAg positive patients were further tested for different serological parameters. Co-infection of HCV and HDV was detected by ELISA. HBV co-infection with HDV was found positive in total of 21 (9.90 %) samples consisting of 11.22 % (n 11) female and 8.77 % (n 10) male respectively. Rate of HBV co-infection with HDV was found more frequent in female as compared to male patients as shown in table no 1.

Table 4.1 Frequency of HBVand HDV co-infection in male and female patients

Gender	Total samples HBV positive	Found Positive with HDV	Percentage
Male	114	10	8.77 %
Female	98	11	11.22 %
Total	212	21	9.90 %

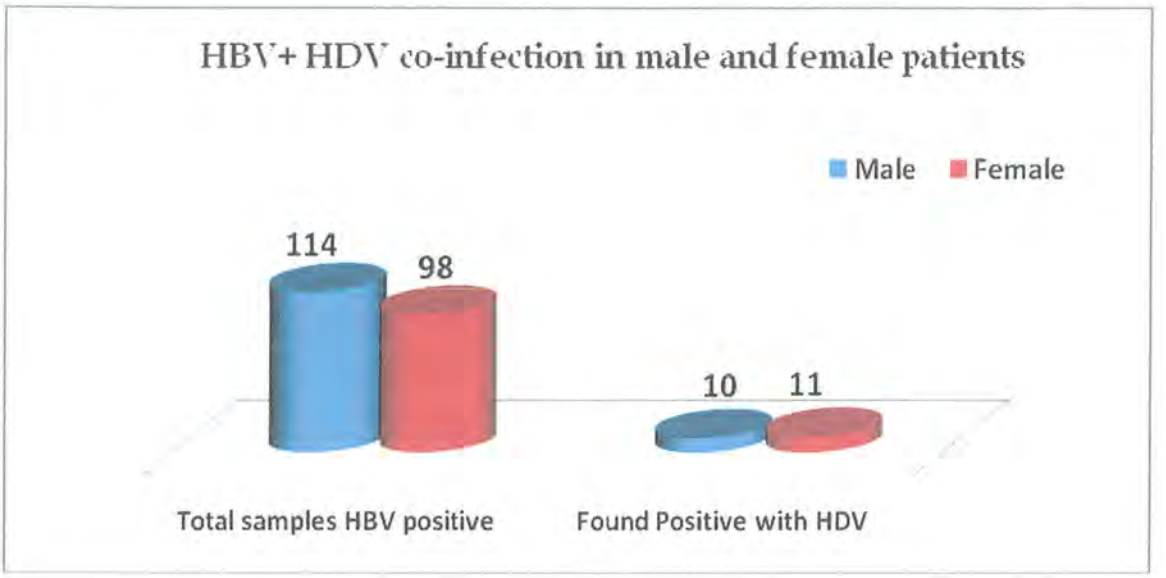

Figure 4.1: Graphical representation of HBV and HDV co-infection in male and female patients

Of 212 HBV infected patients co-infection with HCV was found in 7.54 % (n 16) patients. Male 8.77 % (n 10) were predominantly co-infected with HCV as compared to female 6.12 % (n 6) as shown in table no 2.

Table! 4.2; Frequency of HBVand HCV co-infection in male and female patients

Gender	Total samples HBV positive	Found Positive with HCV	Percentage
Male	114	10	**8.77 %**
Female	98	06	**6.12 %**
Total	**212**	**16**	**7.54 %**

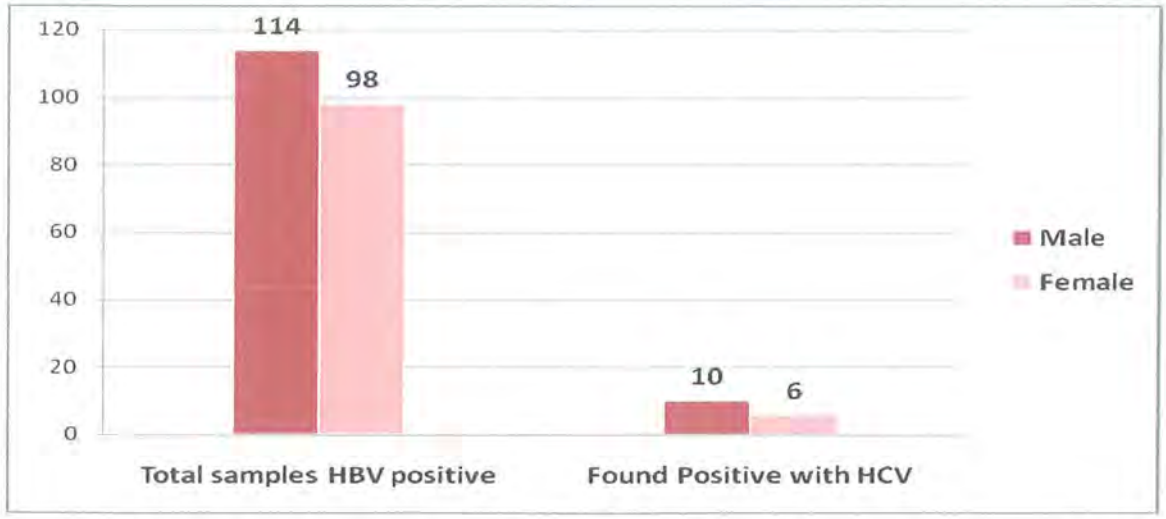

Figure 4.2: Graphical representation of HBV+ HCV co-infection in male and female patients

All HBV positive cases were further screened for serological markers by ELISA and HBV DNA by PCR. Out of 212 patients 184 (86.79 %) were found positive with hepatitis B core total antibody that indicates the rate of chronic infection. Similarly, 25.0 % (n 53) were found positive for hepatitis B core IgM that reflects acute HBV infection. HBV DNA was detected in 24.52 % (n 52) of patients with active DNA replication. Furthermore, co-infection with HDV and HCV was found in 9.90 % and 7.54 % of patients respectively as shown in table no 3.

Table 4.3 Total Positive cases and their respective Percentage

Parameter	Total Cases	Positive	**Percentage**
HBcore Total	212	184	**86.79%**
HBcore IgM	212	53	**25.00%**
Anti-HDV	212	21	**9.90%**
HBV DNA	212	52	**24.52%**
Anti-HCV	212	16	**7.54%**

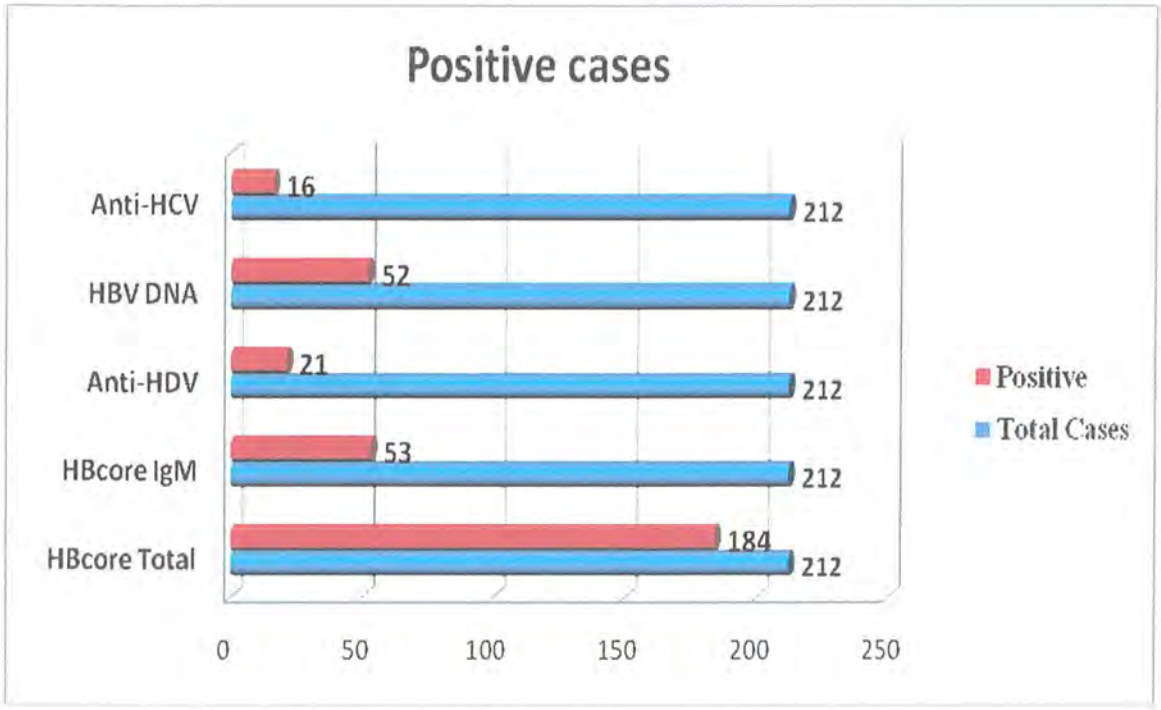

Figure 4.3: Graphical representation of total Positive cases and their respective Percentage

To measure the age-wise frequency, all the HBsAg positive cases were distributed into four groups of ages as shown in (Table 5). It was observed that all the age groups were affected except age group of <5 years that was less commonly infected. The major rate of incidence 70.75% was found in the age group of 5-30 years while a very least incidence of 0.94% and 5.66% was found in the age group of below 5 years and 50+ years (Table 5).

Table 4.4: Age wise distribution of positive Cases and their respective percentage.

S. No	*Ages/ Years*	*Total Cases*	*Positive*	Percentage
1	< 5	212	02	0.94 %
2	05 – 30	212	150	70.75 %
3	31 – 50	212	48	22.64 %
4	50+	212	12	5.66 %

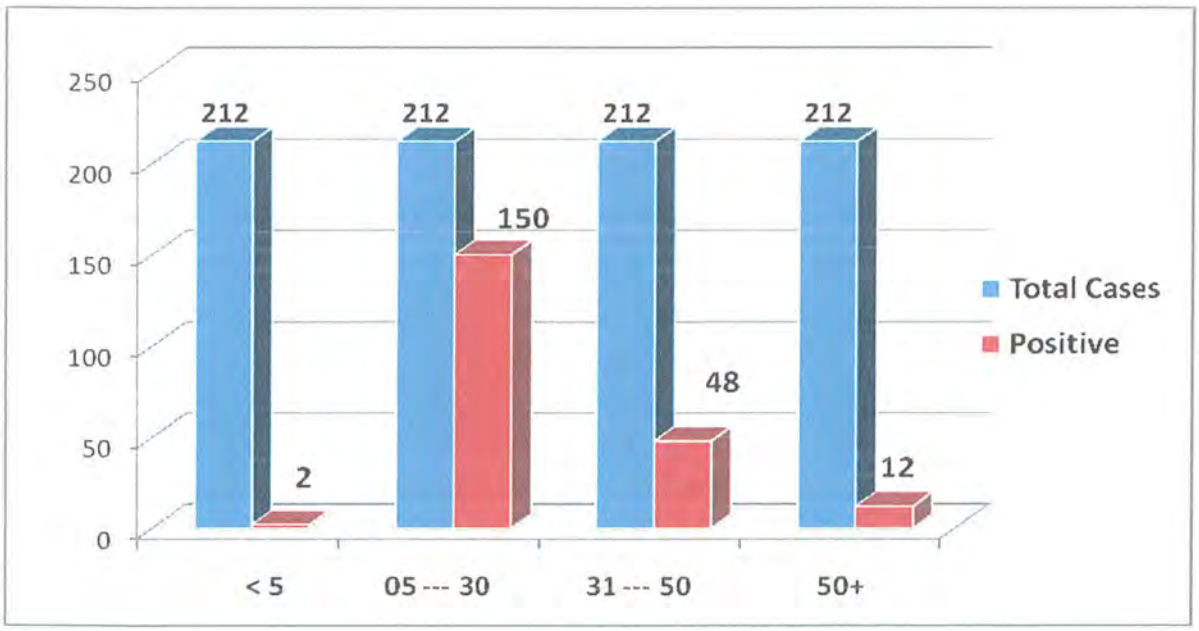

Figure 4.4: Graphical representation of Age wise distribution of suspected and positive Cases.

The incidence frequency of gender among the HBV infected persons showed that more men were infected than the women (Table No.6). In our study HBsAg, HBcore total HBcore IgM, HCV, HDV and PCR positive males were 55.77% (114), 49.05 (104), 17.45% (37), 4.71 (10), 4.71% (10) and 11.79% (25) respectively as compared to female which were less frequently infected.

Table 4.5: Gender wise distribution of confirm cases

Parameter	Male		Female	
	+Ve	%ages	+Ve	%ages
HBsAg	114	**53.77 %**	98	**46.22 %**
HBcore Total	104	**49.05 %**	80	**37.73 %**
HBcore IgM	37	**17.45 %**	16	**7.54 %**
Anti-HDV	10	**4.71 %**	11	**5.18 %**
HBV DNA	25	**11.79 %**	27	**12.73 %**
Anti-HCV	10	**4.71 %**	06	**2.83 %**

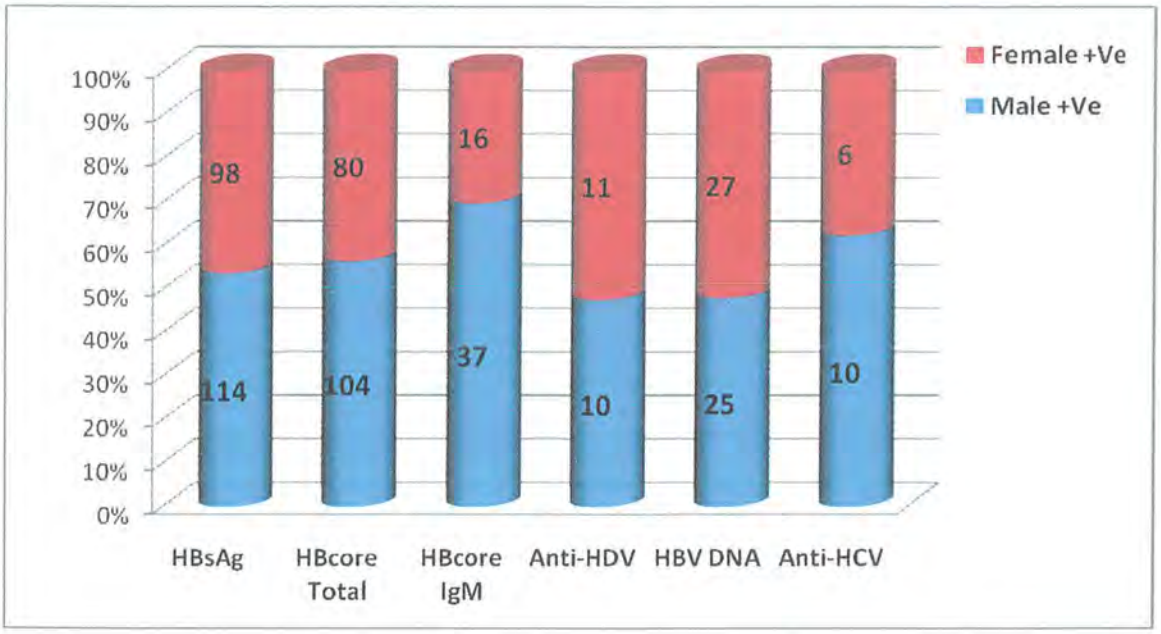

Figure 4.5: Graphical representation of gender wise distribution of confirmed cases

Among the overall positive subjects of HBV, trends of using personal things like shaving razor, needles used for ear and nose piercing, IV needles surgical instruments and dental procedure equipments were very common. This study pointed out that personal contact were the most predominant (66.03%) risk factors among individuals (Table7). In this study, the predisposing factors of B viral hepatitis included risk of past history of dental procedures (39.62%) injections given (87.73%) IV infusion (83.96%), visit to barber (men only) (51.88%), hospitalization (46.69%), skin piercing (41.98%), Surgery (25.47%) visit to beauty parlor (18.49%), blood transfusion (8.01%) illegal injection drug use (7.54%) and tattoos or acupuncture (5.18%) as shown in table 7.

Table 4.6. Risk factors associated with HBV infection.

Risk Factor	Yes	%	No	%
Injections given	186	87.73 %	26	12.26 %
Hospitalization	99	46.69 %	113	53.30 %
Surgery:	54	25.47 %	158	74.52 %
Blood transfusion	17	8.01 %	195	91.98 %
IV infusion:	178	83.96 %	34	16.03 %
Dentist visit:	84	39.62 %	128	60.37 %
Visit to barber (men only):	110	51.88 %	102	48.11 %
Visit to beauty parlor	18	8.49 %	194	91.50 %
Skin piercing:	89	41.98 %	123	58.01 %
Tattoos or acupuncture:	11	5.18 %	201	94.81 %
Illegal injection drug use:	16	7.54 %	196	92.45 %
Household contact	140	66.03 %	72	33.96 %

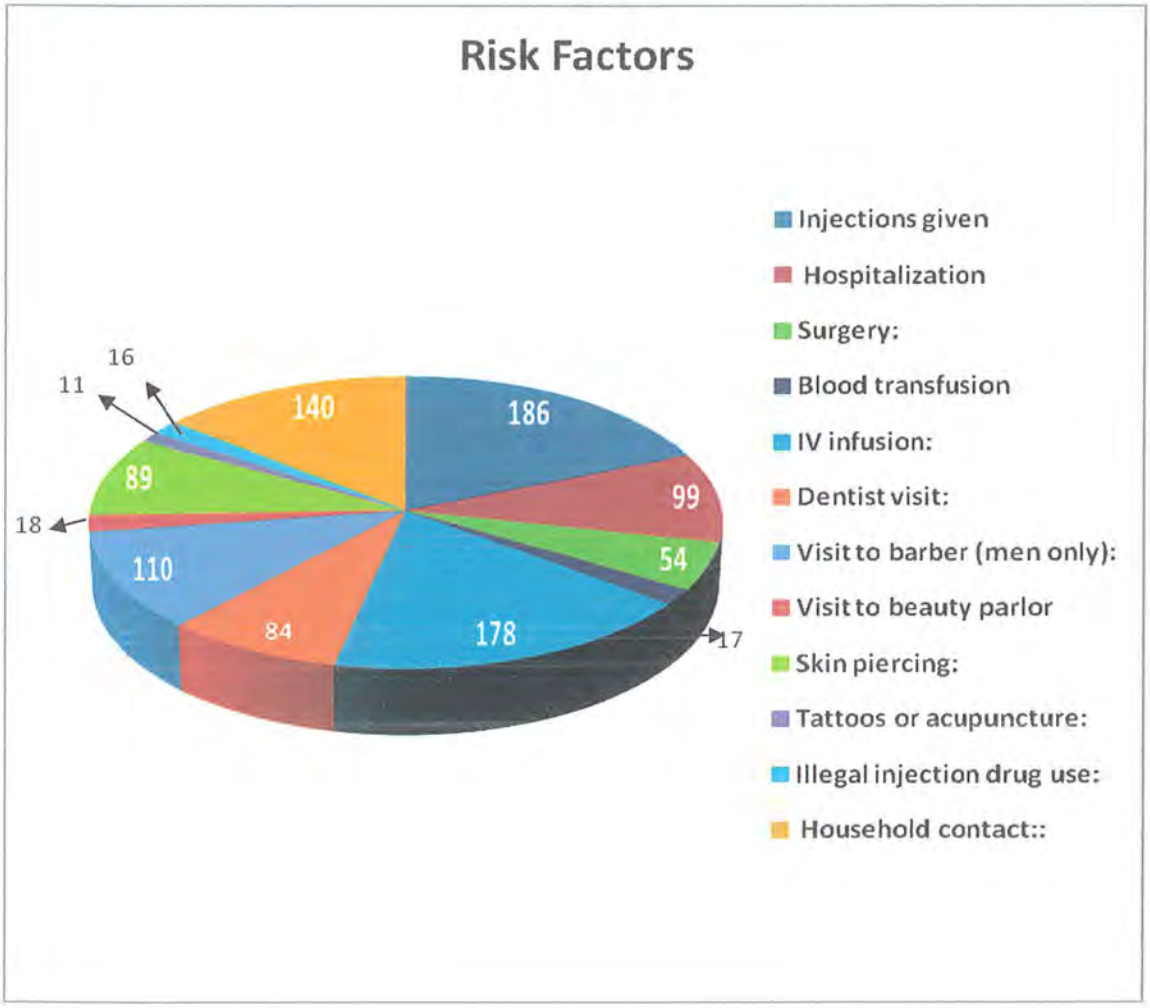

Figure 4.6: Graphical representation of associated Risk factors with HBV infection.

Among the overall HBV positive cases most common symptoms were abdominal discomfort, fatigue anorexia, malaise, fever and vomiting. In this study the main symptoms of B viral hepatitis included abdominal discomfort (79.24%) fatigue (72.64%) anorexia (66.03%) malaise (61.79%) fever (53.77%) vomiting (49.05%) dark urine (44.33%) pain RHC (37.73%) and Splenomegaly (2.35%) were noted as shown in table 8.

Table 4.7; Symptoms associated with HBV infection.

Symptoms	Yes	%	No	%
(1) ❑ Abdominal Discomfort	168	79.24 %	44	20.75 %
(2) ❑ Unknown pain RHC	80	37.73 %	132	62.26 %
(3) ❑ Anorexia	140	66.03 %	72	33.96 %
(4) ❑ Fatigue	154	72.64 %	58	27.35 %
(5) ❑ Fever	114	53.77 %	98	46.22 %
(6) ❑ Malaise	131	61.79 %	81	38.20 %
(7) ❑ Dark urine	94	44.33 %	118	55.66 %
(8) ❑ Splenomegaly	05	2.35 %	207	97.64 %
(9) ❑ Vomiting:	104	49.05 %	108	50.94 %

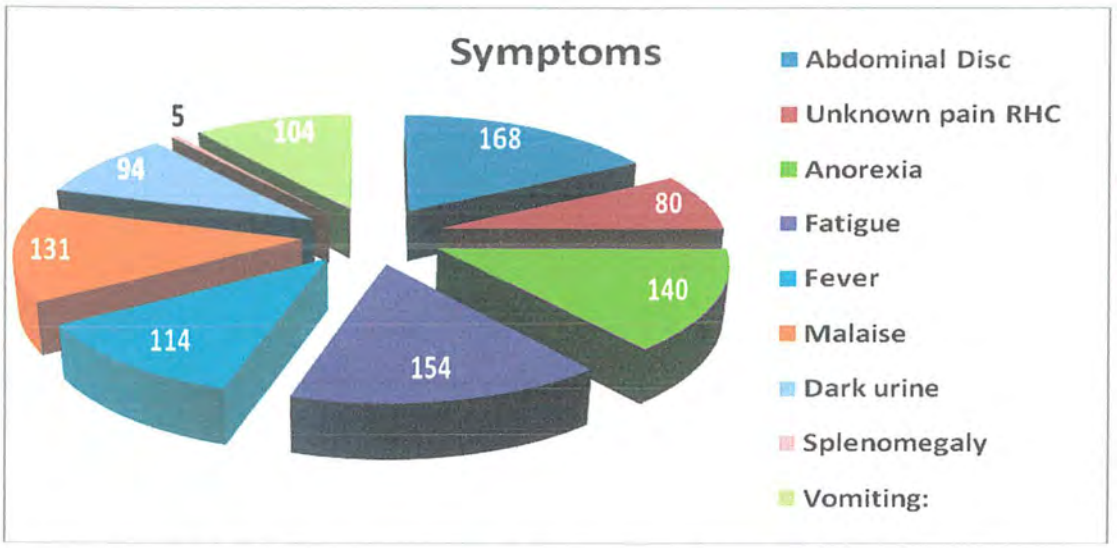

Figure 4.7: Graphical representation of Symptoms associated with HBV infection.

DISCUSSION

B viral hepatitis infection is a critical public health issue in both underdeveloped and developing countries affecting approximately 3.5 billion of population worldwide. Moreover ≥ 400 million are chronic carriers of this disease. In Pakistan, published work about HBV infection has been found which grossly discuss HBV epidemiology and high rate of prevalence. Different factors among published data, like sample size, socio-economic status, diagnostic assays practiced, risk factors associated, community under observation, ethnicity and behavior of general population reflects quite different picture of the disease. The current study briefly describes the incidence rate, risk factors correlated with HBV transmission among both male and female population of different age groups, chronic and acute status of HBV and comparison of different genetic and serological marker of HBV prevalent in KPK population.

Out of total 212 HBsAg, ELISA positive samples, 53.77% (n= 114) were male and 46.22%, (n= 98) were female. Out of 212 HBsAg positive patients n= 184 (86.79%) were found positive with anti-HBcore total indicating high rate of chronic infection. 25.0 % (n= 53) were found positive for hepatitis B core IgM which reflects acute HBV infection. Similarly HBV DNA was detected in 24.52 % (n= 52) of patients with active DNA replication. HBV co-infection prevalence with HDV and HCV was found to be 9.90 % and 7.54 % respectively. Males were most commonly infected than females.

A similar study conducted by Khan *et al.,* (2011) in IDPs population of Malakand division reported 21.05% prevalence of HBV. This study reported that males (78.5%) were infected three times as compared to females (21.5%) with HBV infection. Males are more prone to get infection with HBV due to higher exposure to major risk factors in comparison to females spending most of the time in their homes thus, less exposed to different risk factors. Shazi and Abbas (2006) also reported same results of high incidence of HBV infection in men (78.04 %) and low incidence in women (21.95%). The study was about comparative analysis of risk factors associated with hepatitis B and C infection conducted in a liver stomach clinic Karachi. Mahtab *et al.,* (2008) in

Bangladesh report similar results of prevalence in men (67.86 %) and women (32.14 %). High prevalence in males defined the escalating frequency of exposure to high risk practices as compared to females which might include multiple sexual partners, drugs abuser and visit to barber.

The present study confirmed that all the age groups were influenced equally except age group <5 years being less commonly infected. The highest incidence rate of 70.75% was detected in the age group of 5-30 years while very low incidence of 0.94% and 5.66% was observed in the age group <5 years and >50 years. Very young and old people were not instantly infected by HBV. Alam *et al.,* (2007) also found that n=52, (4%) patients were encounter positive for HBsAg, and their mean age was 23.5 ± 3.7 years. But 30-40 years age group was determined with sharp frequency of HBV infection. Khan *et al.,* (2011) found that majority of the infected people were within the age group of 21-30 (34.93%) simultaneously followed by 23.83% in age group of 31-40. It was further reported that only (13.39%) were patients of ages between 11-20 years. The advance prevalence among teenage groups may account for recurrent exposure to risk factors and may prolong HBV infection. But low prevalence was noted in children of age <5 years , might be due to vaccination for B viral hepatitis as a part of EPI being introduced in 2004 countrywide vaccination campaign. Extraordinary consideration was given to newborn under 1 year of age.

Among HBV assured individuals, practice of sharing different kinds of personal items like razor blades used during Barber visit, nose and ear penetrating needles, IV needles surgical instruments and dental procedure equipment were common. This study declared that personal contact was the utmost (66.03%) risk factors recognized in these patients. In this study the predisposing factors of B viral hepatitis comprised of earlier risks of dentist procedures (39.62%), injections given (87.73%), IV infusion (83.96%), visit to barber (men only) (51.88%), hospitalization (46.69%), skin piercing (41.98%), Surgery (25.47%), visit to beauty parlor (18.49%), blood transfusion (8.01%), illegal injection drug use (7.54%) and tattoos or acupuncture (5.18%).

Khan *et al.*, (2011) concluded that barber visit is a major risk of HBV transmission and its exposure frequency was clearly greater (32%) in male patients who were consistently shaved by local barbers. Janjua and Nizamy (2004) narrated that about 46% of the hairdresser in Pakistan repeatedly shaved with unchanged blades, thus increasing the chance of transmission of HBV. Komas *et al.*, (2010) concluded that socioeconomic conditions, sexual activity and familial antecedents of HBV were the capital risk factors of HBV infection experienced in the teenagers.

Usman *et al.*, (2003) reported high percentage (60 %) of HBV patient with past history of dental surgery which is distinguished from current study. While 25.47% patients positive with past history of surgical procedure is an additional risk factor of B viral infection in this study. Khan *et al.*, (2007) and Shazi and Abass (2006) also narrated closely matched results of 21.43 % and 21.9 % related to surgical procedures. Majority of the infected individuals were from impaired economical backgrounds and were treated by local medical practitioners. In reality these practitioners mostly do not have any knowledge about sterilization of medical equipments. Due to the lack of sterilization approach, reuse of disposable needles and continuous use of contaminated equipment high rate of prevalence might be found in these areas.

In this study HBV co-infection with HDV was found positive in a total of 21 (9.90 %) consisting of 11.22 % (n=11) female and 8.77 % (n=10) male respectively. Khan *et al.*, (2011) reported high prevalence of HDV co-infection in Sindh province and found HDV RNA in n=53 (28%) patients out of 190 subjects which were confirmed HBV DNA positive. They further classified these 53 HDV positive cases into male, n=37 (69.8%) and female which were n=16 (30.2%) respectively. Another study of HDV co-infection in HBV infected individuals was conducted by Shaikh *et al.*, (2012) in Larkana. They reported that out of 1564 HBV DNA detected serum samples, the HDV RNA was detected in n=865 (55.31%) and not detected in n=699 (44.69%) individuals. Zaidi *et al.*, (2010) reported maximum prevalence tendency of HDV infection in Punjab Pakistan. They reported that Out of 96 B surface Ag ELISA positive cases, n=80 (88.8%) were antibodies to HDV positive and among these n= 24 (30%) were positive by RNA of

HDV. Delta virus super-infection was more casual in men patients than women patients (81% to 19%). The results of this study further proposed that the prevalence of HBV/HDV co-infection in KPK is much lower as compared to Sindh and Punjab. Worldwide HDV infection observed to be decline. HBV vaccination and treatment may be the reason of decline in HDV prevalence internationally. One other reason of low prevalence might be variation in technique used during performing of test. Interestingly very limited work has been done on HDV co-infection in KPK to clarify true prevalence.

In this study out of 212 HBV infected patients co-infection with HCV was found in 7.54 % (n=16) patients. Male 8.77 % (n=10) were predominantly co-infected with HCV as compared to female 6.12 % (n=6). Shaikh *et al.*, 2012 reported that out 1713 patients, anti-HCV Ab was detected in 420 (24.5%), anti-HDVAb in 1116 (65.1%). Zuberi *et al.*, (2008) reported that out of the 246HBV infected patients, 29 (11.8 %) cases were also positive for IgG antibodies to HBcore, anti-HDV and anti-HCV. Tadjiev (2010) detected mixed infection of HBV+ HCV in n=33 (35.1%) patients of Tashkent Pediatric Medical Institute Uzbekistan. Mathurin *et al.*, (2000) reported 32% patients had TH (HBV, HDV, HCV) and multiple infections were related with a decline in HCV replication and cirrhosis was more instantly observed in individuals with multiple infections.

Zhou *et al.*, (2012) reported multiple co-infections among injecting drug abusers in Yunnan province of China. They found 15 % of HIV/HCV, 0.3 % of HIV/HBV, 7.8 % of HCV/HBV and 7.1% of HIV/HCV/HBV co-infection respectively. Another study of co-infection was conducted by Gholamreza *et al.*, (2007) in Iranian population and they reported that out of 139 HBV positive cases n=8 (5.8%) were infected with HDV and n=17 (12.3%) subjects were positive with anti-HCV. But in current study males were more frequently infected than female. High prevalence in males represents the increased frequency of high risk behavior and exposure to the outside environment.

Conclusions

The observed prevalence is age and gender-dependent which might be due to their increasing exposures to general risk factors. In KPK majority of people have chronic hepatitis B infection as compare to acute Hepatitis B infection. To prevent the transmission of B viral infection considerable awareness about the possible common risk factors and immunization extension is recommended. Convincing hygienic manner should be utilize during dental and surgical procedure. PCR based test can detect low level of viraemia in non-replicative HBV disease. The HBV co-infection with HCV and HDV is not uncommon in Pakistan that tends to increase over time. Primarily public health authority should take purposeful initiative to educate and create awareness of people about various risk factors to prevent the transfer of B viral infection and impose surveillance and immunization program to backward regions.

Recommendations

This study signifies the need for heightened attention and preventive measures against the infection of hepatitis. Innovative approaches for HBV immunization inflation among the rural people and enforcement of Hepatitis surveillance system. HBV Precore mutation, drugs resistance and Genotyping are the future prospective of among infected individuals.

Future prospects

- Genotyping of HBV.
- Host viral interaction .
- Identification of Polymorphism MBP.
- Prospect for study of HBV resistance allele.
- Alternative drug strategy method for treatment of HBV.
- Genetic predisposition.

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Questioner for HBV Infection

PATIENT DEMOGRAPHIC INFORMATION			
Date of report: ____ / ____ /_____		Patient/guardian NID :	
Patient name:	S/O,W/O,D/O:		Date of birth:
Address:		Phone No:	
Gender: 1 ❑ Male 2 ❑ Female		Occupation:	Education:
Contact: Name	Relation	Age	

CLINICAL CHARACTERISTICS			
Jaundice:	1 ❑ Yes 2 ❑ No	Date of jaundice onset	____ / ___ /_____
Hospitalization:	1 ❑ Yes 2 ❑ No	Date of hospitalization:	____ / ___ /_____

CLINICAL INFORMATIONS

(1) ❑Abdominal Discomfort **(2)** ❑Unknown pain RHC **(3)** ❑ Anorexia **(4)** ❑Fatigue
(5) ❑ Fever **(6)** ❑Malaise **(7)** ❑ Dark urine **(8)** ❑Splenomegaly **(9)** ❑Other::

PLEASE READ: **"The following questions refer to exposures in the <u>six months</u> before onset"**

Vaccinated against hepatitis B ❑ Yes(year_____) ❑ No ❑ Unknown

Contact with jaundiced person: 1❑ Yes 2 ❑ No 3 ❑ Unknown

Water source for drinking or cooking:
1 ❑ Municipal 2 ❑ Bottle 3 ❑ Well water – treated
4 ❑Well water untreated 5 ❑ Surface water 6 Other:_____

Do you boil water for drinking? 1 ❑ Yes 2 ❑ No 3 ❑ Unknown

(1). Injections given Hospitalization: **(2)**. Surgery: **(3)**. Blood transfusion:
(4) IV infusion: **(5)** Dentist visit: **(6)** Visit to barber(men only):
(7) Visit to beauty parlor (women only): **(8)** Skin piercing: **(9)** Tattoos or acupuncture:
(10) Illegal injection drug use: **(11)** Religious body stabs::
(12) Spouse status: HIV, Hepatitis B/C):: **(13)** Status of mother in case of young children(HIV, Hep B/C)

LABORATORY RESULTS (SPECIMEN COLLECTON DATE: ____ / ___ /_____)

(1) ALT:_____U/L (2) AST: _____U/L (3) ALP: ____U/L (4) Bilrubin: _____mg/dl
HBs Ag: Anti HBc Total: IgM anti-HBc:
Anti-HDV: HBV DNAQualitative:

CLINICAL CHARACTERISTICS

Medical officer (print):_____ Date completed: _____ /_____ /_____
Medical unit (where patient interviewed): **1** ❑ OPD 2 ❑ Indoor patient 3 ❑ Other_____

FOR SURVEILLANCE OFFICER USE ONLY

Surveillance officer Name: Date:
Comments